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Reverse chemical ecology approach for the identification of an oviposition attractant for *Culex quinquefasciatus*

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Pheromones and other semiochemicals play a crucial role in today's integrated pest and vector management strategies. These semiochemicals are typically discovered by bioassay-guided approaches. Here, we applied a reverse chemical ecology approach; that is, we used olfactory proteins to lead us to putative semiochemicals. Specifically, we used 7 of the top 10 odorant receptors (ORs) most expressed in the antennae of the southern house mosquito, *Culex quinquefasciatus*, and which are yet to be deorphanized. We expressed these receptors in the *Xenopus* oocyte recording system and challenged them with a panel of 230 odorants, including physiologically and behaviorally active compounds. Six of the ORs were silent either because they are not functional or a key odorant was missing. CquiOR36, which showed the highest transcript levels of all *OR* genes in female antennae, was also silent to all odorants in the tested panel, but yielded robust responses when it was accidentally challenged with an old sample of nonanal in ethanol. After confirming that fresh samples were inactive and through a careful investigation of all possible "contaminants" in the old nonanal samples, we identified the active ligand as acetaldehyde. That acetaldehyde is activating CquiOR36 was further confirmed by electroantennogram recordings from antennae of fruit flies engineered to carry CquiOR36. Antennae of female mosquitoes also responded to acetaldehyde. Cage oviposition and dual-choice assays demonstrated that acetaldehyde is an oviposition attractant in a wide range of concentrations and thus of potential practical applications.

southern house mosquito | CquiOR36 | acetaldehyde | odorant receptors | EAG

Since the chemical identification of the sex pheromone of the silkworm moth bombykol almost six decades ago (1), chemical ecologists have been identifying semiochemicals involved in intraspecific (e.g., sex pheromones), interspecific (e.g., kairomones), and tritrophic interactions and exploring their potential use in agriculture and medical entomology. These researchers have developed semiochemical-based environmentally friendly strategies for monitoring and controlling populations of agricultural pests and insects of medical importance, including trapping systems for monitoring and surveillance, mating disruption, and attraction-and-kill systems. In agriculture and medical entomology, these semiochemicals are applied in combination with other strategies in Integrated Pest Management (IPM) and Integrated Vector Management (IVM), respectively. IVM, for example, is a rational decision-making process to make vector control more efficient, cost-effective, ecologically sound, and sustainable. It is ultimately aimed at preventing the transmission of vector-borne diseases (2). IPM also is aimed at reducing transmission of pathogens to plants, but its main focus is on reducing populations of harmful insects to below economic thresholds. One of the IVM cornerstones is vector surveillance, which is a systematic monitoring of the seasonality and abundance of vector populations both to ensure appropriate and timely interventions and to evaluate the effect of vector control (2). Throughout the United States, hundreds of vector abatement districts (67 agencies in California alone) are constantly engaged in vector surveillance not only to monitor populations of native species and the circulation of pathogens, but also for quarantine of invasive mosquito species as well as to monitor circulation of new and previously reported pathogens. In addition to labor-intensive strategies, such as sampling of immature stages and aspiration of adult mosquitoes from house to house, abatement district personnel rely heavily on capturing host- and oviposition-seeking mosquitoes with surveillance traps. Although carbon dioxide is the most effective lure, CO_2-baited traps capture blood-seeking mosquitoes and thus are less effective for early detection of a pathogen because they trap many mosquitoes that have never had a blood meal. By contrast, gravid traps are more effective for surveillance because they target a critical epidemiological stage—the gravid females that drank and digested at least one blood meal and therefore are more likely to be infected with a vector-borne pathogen than the general adult population (3). Additionally, ovitraps can also be used as trap-and-kill systems for direct control of mosquito populations. Almost all female mosquitoes trapped in gravid traps have had at least one blood meal, which increases the chances of detection of circulating viruses. Moreover, trapping infected mosquitoes reduces possible virus horizontal and vertical transmission in a subsequent

Significance

Surveillance of mosquito populations is essential for determining the best timing for intervention as well as for monitoring circulation of new and previously detected pathogens. Trapping gravid females is more effective because they are more likely to be infected than nongravid females. However, better gravid trap attractants are sorely needed to replace fermentation recipes, which are cumbersome, have poor quality control, generate an offensive smell, and do not provide a long-term and consistent source of attractants. By using a reverse chemical ecology approach based on odorant receptors from the southern house mosquito, we have identified that acetaldehyde in a wide range of doses both attracts gravid female mosquitoes and stimulates them to lay eggs in oviposition trays.

Author contributions: Y.-M.C., P.X., J.K.H., F.Z., K.T., G.B., and W.S.L. performed research; K.R.C. contributed new reagents/analytic tools; Y.-M.C., P.X., J.K.H., F.Z., and W.S.L. analyzed data; and W.S.L. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. MG214256).

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gonotrophic cycle. Although excellent progress has been made in improving trap design (see examples in refs. 4–6) and new attractants have been identified (see examples in refs. 7–9), better lures are still needed. Typically, abatement districts prepare their own fermentation recipes, which are cumbersome, have poor quality control, generate an offensive smell, and do not provide a long-term and consistent source of attractants. Since early on, several attempts have been made to isolate natural products from infusions and other natural sources (10).

To identify semiochemicals for possible applications in agriculture and medical entomology, chemical ecologists apply bioassay-guided approaches for the isolation of active ingredients, which may be augmented with electrophysiological approaches, such as gas chromatography-electroantennographic detection (GC-EAD). Although GC-EAD may provide a "shortcut" to the active ingredients, a solid and consistent bioassay is still needed to avoid false-positives (a compound may generate an electrical signal and be behaviorally inactive). The explosive advancement in our understanding of the molecular basis of insect olfaction in the last two decades has opened the door for reverse chemical ecology, a term coined for the screening of olfactory proteins as another "shortcut" toward active semiochemicals (11, 12). Two major proteins may be used for such screenings, that is, the odorant-binding proteins (OBPs) and odorant receptors (ORs). OBPs are involved in the transport of odorants through the sensillum lymph surrounding the dendrites of olfactory receptor neurons where ORs are embedded (13). ORs form ion channels with the obligatory and well-conserved coreceptor Orco, which was initially found in the fruit fly and then named OR83b (14). Semiochemicals activating OR-Orco complexes generate electrical signals that are transmitted to the brain and may ultimately lead to a behavioral response. As with GC-EAD, evidence of binding to an OBP or activation of an OR is not sufficient to infer behavioral activity. Activity must be confirmed by indoor and/or field assays. Here, we applied reverse chemical ecology in an attempt to identify semiochemicals of possible applications for monitoring or controlling populations of the southern house mosquito *Culex quinquefasciatus*. First, we identified the top 10 most-expressed *OR* genes in female antennae (15, 16), and then we used 7 *ORs* that are yet to be deorphanized, expressed them in the *Xenopus* oocyte recording system, and tested them with a panel of 230 odorants, including physiologically and behaviorally active compounds. Six of the tested *ORs* were silent to all tested compounds, but one of them, CquiOR36, gave the strongest responses that we have ever recorded from insect ORs to a mistakenly applied old sample of nonanal in ethanol. A careful chemical investigation showed that CquiOR36 was responding to acetaldehyde. This was further confirmed by electroantennogram (EAG) recordings of flies carrying CquiOR36 and mosquito antennae. Behavioral assays showed that acetaldehyde is an oviposition attractant at a wide range of doses and thus has potential practical applications as a lure for gravid traps.

Results and Discussion

Expression Levels of the Top 10 *OR* Genes in *Cx. quinquefasciatus* Genome. Previously, we identified by RNA-Seq *OR* genes that are significantly enriched in the antennae of the southern house mosquito compared with the legs (15) and validated our findings by analyzing expression of the top five *OR* genes by quantitative PCR (qPCR), which yielded transcription data in reasonable agreement with RNA-Seq analysis (15). Because the goal of this research was to complete deorphanization of the top 10 *ORs* and use them for reverse chemical ecology, we first ran qPCR with the top 10 *ORs* as selected by RNA-Seq data (15). Again, the expression pattern of most *OR* transcripts was in agreement with the RNA-Seq data. However, one *OR* in particular, CquiOR36, showed by far the highest expression levels, thus ranking number 1 by qPCR (*SI Appendix*, Fig. S1). We missed this discrepancy in our initial validation because this gene ranked as the sixth in our RNA-Seq analysis (15). Indeed, CquiOR36 expression levels by qPCR are at least threefold higher than that of CquiOR125, the OR previously ranked as number one in the *Cx. quinquefasciatus* genome (15). Next, we compared expression of CquiOR36 in olfactory and nonolfactory tissues and observed that this OR is expressed mainly in antennae, with very low levels in maxillary palps, proboscis, and legs (Fig. 1*A*). Moreover, CquiOR36 is expressed more in female than in male antennae (Fig. 1*B*). When comparing expression of olfactory genes in male and female antennae, one complication is that males have fewer olfactory sensilla than females have because olfactory sensilla are restricted to the 2 distal flagelomeres in males and distributed in all 13 flagelomeres in females (17). One way to circumvent this problem is to normalize expression using Orco as a reference. When normalized to *CquiOrco*, expression levels of *CqOR36* transcripts in female antennae were 3.74-fold higher (3.74 ± 0.12; mean ± SEM) than in male antennae. Of the top 10 OR genes, CquiOR36, CquiOR125, CquiOR151, CquiOR64, CquiOR55, CquiOR132, and CquiOR93 have not been deorphanized (15, 16). By contrast, CquiOR21 (formerly CquiOR10), CquiOR95, and CquiOR136 were previously demonstrated to respond to oviposition attractants (18), ethyl 2-phenylacetate and citronellal (15), and synthetic repellents (16), respectively.

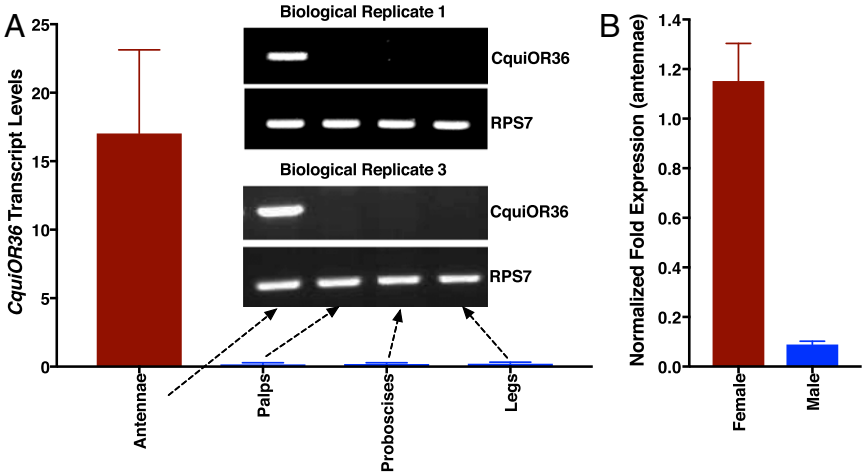

Fig. 1. Quantitative PCR data. (*A*) Transcript levels of CquiOR36 in olfactory and nonolfactory tissues from *Cx. quinquefasciatus* females. For reference, gels obtained before qPCR analysis are displayed. (*B*) Comparative expression of *CquiOR36* in male and female antennae.

Deorphanization Attempts. Oocytes coexpressing CquiOR125 (GenBank accession no. KM229531), CquiOR151 (accession no. KM229534), CquiOR64 (accession no. KM229532), CuiOR55 (accession no. KM229536), CuiOR132 (accession no. KM229535), or CquiOR93 (accession no. KM229533) along with CquiOrco did not respond to any odorants in our test panel. It is not possible to conclude whether these receptors are sensitive to other compounds not included in our panel or even whether their heteromeric channels (with Orco) were properly formed. There are no current methods to unambiguously determine where heteromeric (Or·Orco) channels are formed in *Xenopus* oocytes when they failed to be activated by tested odorants. In most cases, heteromeric OR complexes are more sensitive to Orco agonists than to Orco homomers (19). Two of the best Orco agonists that may be used to infer proper channel formation are *N*-(4-ethylphenyl)-2-[(4-ethyl-5-pyridin-3-yl-1,2,4-triazol-3-yl)sulfanyl]acetamide (VUAA-1) (20) and 2-(4-ethyl-5-(4pyridinyl)-4H-1,2,4-triazol-3yl)sulfanyl)-*N*-(4-isopropylphenyl)-acetamide (OLC-12) (21). CquiOR55·CquiOrco-expressing oocytes were more sensitive to VUAA-1 than Orco homomers, thus suggesting that this receptor is functional, but the right ligands are missing in our panel. By contrast, oocytes expressing CquiOR125, CquiOR151, CquiOR64, CquiOR132, or CquiOR93 along with CquiOrco, did not elicit detectable currents when challenged with VUAA-1. We then challenged these oocytes with OLC-12 and recorded stronger responses than those elicited by VUAA-1 at the same dose (0.01 mM) in oocytes expressing CquiOR55 along with CquiOrco. We also recorded detectable currents elicited by OLC-12 on oocytes coexpressing CquiOrco with CquiOR93, CquiOR125, or CquiOR132, but CquiOR151·CquiOrco- and CquiOR64·CquiOrco-expressing oocytes were also silent to OLC-12. The receptor with highest expression levels in female antennae, CquiOR36, responded to both VUAA-1 and OLC-12 when coexpressed with CquiOrco in *Xenopus* oocytes. Previously, we considered CquiOR36 as a possible pseudogene (16), but we have now succeeded in cloning the full-length sequence of this gene by using a low annealing temperature and short primers to avoid hairpin formation. Our sequence (MG214256) differs from the sequence in VectorBase (CPIJ004162), which misses 36 internal amino acid residues. Of note, the CquiOR36 full-length sequence is contained in TCONS34486 from a previous RNA-Seq analysis (15). We therefore concluded that there could be an error in the annotation for this gene in VectorBase.

Unusual Response of CquiOR36·CquiOrco-expressing Oocytes. Although CquiOR36·CquiOrco-expressing oocytes were silent to our large panel of odorants, we recorded a robust current when these oocytes were mistakenly stimulated with an old solution of nonanal in ethanol. Typically, we use dimethyl sulfoxide (DMSO) as solvent, particularly in case of aldehydes that may react with the solvent and form hemiacetals. However, this was a fortuitous mistake. The current elicited by this old sample was the strongest current that we have ever recorded from oocytes, including the robust currents elicited by pheromones on pheromone receptors (22, 23). We then prepared fresh nonanal solutions using DMSO or ethanol as solvents. CquiOR36·CquiOrco-expressing oocytes were challenged with nonanal samples left at room temperature for 3 d and freshly prepared samples in these two solvents (*SI Appendix*, Fig. S2). These oocytes did not respond to fresh and old nonanal samples in DMSO, but again generated robust responses to old samples in ethanol and minute responses to freshly prepared nonanal samples in ethanol (*SI Appendix*, Fig. S2). Analysis of the nonanal samples by gas chromatography-mass spectrometry showed a small contamination with nonanoic acid. We then surmised that nonanoic acid, not nonanal, might be activating the receptor. Freshly prepared samples of nonanoic acid in DMSO did not elicit currents in the oocytes (*SI Appendix*, Fig. S3). Next, we tested whether hemiacetals (formed by reaction of nonanal with ethanol) might be activating the receptor. Nonanal solutions in ethanol or DMSO were kept at different conditions and tested with the same oocyte preparations. Freshly prepared samples (in ethanol) and those kept at −20 °C for 24 h elicited currents just above background levels. Of note, frozen samples had to be brought to room temperature before use. Samples kept at 4 °C, room temperature, and at 37 °C generated increasing responses in this order (*SI Appendix*, Fig. S4). By contrast, none of the nonanal samples in DMSO kept under the conditions above generated significant currents (*SI Appendix*, Fig. S4). We inferred that the receptor could be responding to 1-ethoxynonan-1-ol, the hemiacetal generated by reaction of ethanol with nonanal. Because hemiacetals are unstable intermediates and, therefore, cannot be isolated, we synthetized four stable compounds having moieties resembling the structure of 1-ethoxynonan-1-ol, specifically, 3-heptyltetrahydrofuran-2-ol, 3,3-diheptyltetrahydrofuran-2-ol, 3-heptyl-5-methyltetrahydrofuran-2-ol, and 3,3-diheptyl-5-methyltetrahydrofuran-2-ol (*SI Appendix*, Fig. S5). None of these compounds elicited currents in the oocytes coexpressing CquiOR36 and CquiOrco (*SI Appendix*, Fig. S5), but these oocyte preparations responded to VUAA-1 and generated robust currents in response to 3-d-old samples of nonanal in ethanol. Next, by using solid-phase micro extraction (SPME), we analyzed old samples of nonanal in ethanol in an attempt to identify other possible contaminants. In addition to nonanal and ethanol, we detected small peaks of nonanal and acetaldehyde. We then challenged CquiOR36·CquiOrco-expressing oocytes with samples of a series of aldehydes from 1 to 10 carbons, which were dissolved in DMSO.

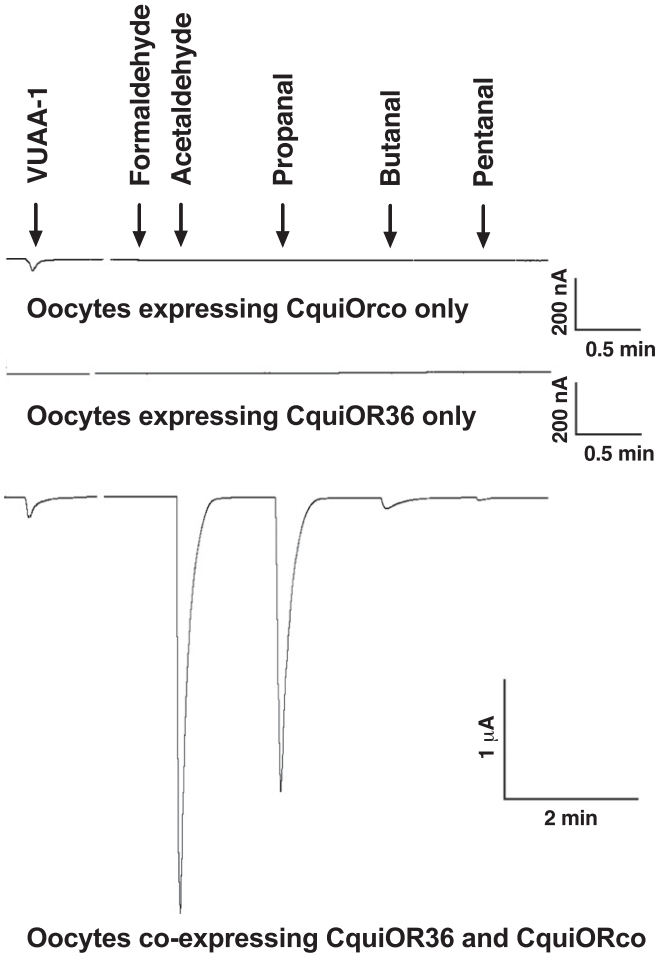

Fig. 2. Responses of oocytes to an Orco agonist and to a series of aldehydes. Oocytes expressing Orco only (top trace) responded to VUAA-1, but not to the aldehydes, whereas oocytes expressing CquiOR36 only (middle trace) did not respond to any tested compound. By contrast, CquiOR36·CquiOrco-expressing oocytes (lower trace) yielded robust responses to acetaldehyde and strong responses to propanal. The response to the Orco agonist was somewhat stronger in the oocytes expressing the heteromeric OR complex than the Orco homomer.

Acetaldehyde samples elicited robust responses (*SI Appendix*, Fig. S6). Propanal also generated strong responses, but none of the other aldehydes in the series, including nonanal and decanal, elicited significant responses (*SI Appendix*, Fig. S6). To ascertain that aldehyde-elicited currents were derived from CquiOR36·CquiOrco channels, we tested oocytes expressing CquiOrco only, CquiOR36 only, and those coexpressing CquiOR36 and CquiOrco (Fig. 2). Indeed, acetaldehyde and, to a lesser extent, propanal generated currents only when CquiOR36 was coexpressed with CquiOrco (Fig. 2).

EAG Responses of Flies Expressing CquiOR36. Using a previously reported assay (24), we expressed CquiOR36 in the fruit fly and recorded EAG responses. Flies overexpressing CquiOR36 in antennae (UAS-CquiOR36/DmelOrco-Gal4) generated robust, dose-dependent responses to acetaldehyde, whereas UAS-CquiOR36/+ flies generated minor responses even when challenged with the highest doses tested (1%) (Fig. 3). Likewise, mosquito antennae responded to acetaldehyde (*SI Appendix*, Fig. S7), thus confirming by three independent methods that both CquiOR36 is sensitive to acetaldehyde and that this semiochemical is electrophysiologically significant for mosquitoes.

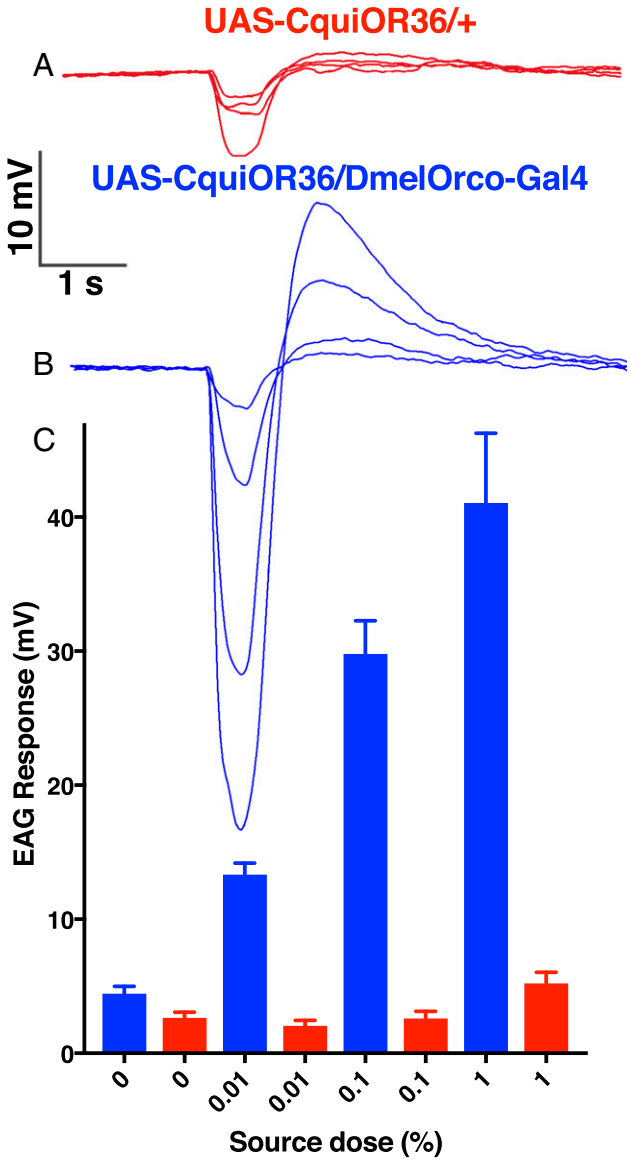

Fig. 3. EAG responses to acetaldehyde recorded from the fruit fly antennae. (*A* and *B*) Traces obtained with UAS-CuiOR36/+ and UAS-CquiOR36/DmelOrco-Gal4 flies when challenged with 0 (solvent only), 0.01, 0.1, and 1% acetaldehyde (from top to bottom). (*C*) Graphic representation of repetitions (n = 3–4) of above experiments. Error bars represent SEM.

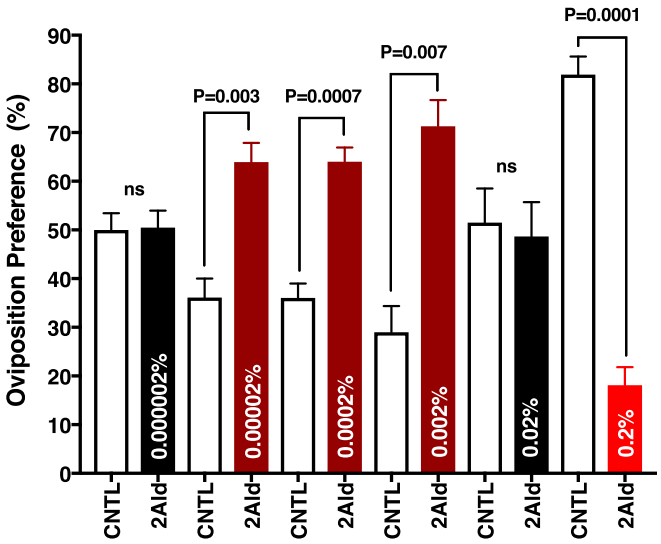

Fig. 4. Behavioral response from gravid females of the southern house mosquitoes to acetaldehyde in a cage oviposition assay. Each pair of bars represents one experiment with two choices: acetaldehyde (2Ald) vs. control (CNTL). Error bars represent SEM. From left to right, n = 15, 15, 16, 11, 16, and 13. Concentrations are indicated inside treatment bars. Data were analyzed with Wilcoxon matched-pair signed-rank tests.

Molecular-Based Insight on Possible Biological Function. Gene expression studies indicated that *CquiOR36* is by far the most-expressed OR in *Cx. quinquefasciatus* antennae (*SI Appendix*, Fig. S1) and that expression is restricted to female antennae (Fig. 1). Female mosquitoes rely on semiochemicals to find plants (to acquire energy for flight) and vertebrates (for a blood meal) and to locate oviposition sites. To get a better insight into how to measure mosquito behavior in response to acetaldehyde, we analyzed gene expression in an attempt to determine whether CquiOR36 is relevant for host- and blood-seeking behavior and/or oviposition behavior. We studied groups of female mosquitoes from the same cohorts, with each batch being separated into two groups. One group of 5-d-old mosquitoes received two blood meals, whereas the nonblood-fed group continued to be fed on sugar only. One, 4, and 6 d after the time of the blood meal, samples from the two groups were prepared for qPCR for comparison. Six days after the time of the blood meal, transcript levels of *CquiOR36* dropped dramatically in the groups of mosquitoes not having a blood meal (*SI Appendix*, Fig. S8), but remained unchanged in blood-fed mosquitoes that were ready for oviposition. These findings suggest that detection of acetaldehyde might be very important for oviposition behavior, but these analyses do not rule out the possibility that acetaldehyde could also be detected in a different context. We tested these two possibilities by using behavioral measurements.

Behavioral Studies. Using a standard cage oviposition assay (9) and testing acetaldehyde at decadic dilutions from 0.2 to 2×10^{-6}%, we observed that *Cx. quinquefasciatus* females laid significantly more eggs in trays baited with acetaldehyde than in control (water only) trays (Fig. 4) at certain doses. At a very low dose (2×10^{-6}%), there was no significant difference between treatment and control. Responses in treatments were highly significant at doses from 2×10^{-5} to 2×10^{-3}%, but at a higher dose (0.02%) acetaldehyde was not active (Fig. 4). A clear oviposition

deterrent effect was observed at an even higher dose (0.2%). Although very convenient, the cage oviposition assay measures the "end point" of the oviposition behavior and does not allow us to tease apart attraction from stimulus. Measurement of behavior in an improved version of our dual choice olfactometer (25) clarified that acetaldehyde is indeed an oviposition attractant and not an attractant for blood-seeking females. Gravid females showed a highly significant preference for the arm of the olfactometer delivering acetaldehyde at 0.002% dose than for the control arm (Fig. 5*A*). By contrast, blood-seeking females tested under the same conditions did not discriminate between the treatment and test sides of the arena (Fig. 5*B*). These datasets demonstrate not only that the olfactometer is unbiased, but also that blood-seeking mosquitoes are not attracted to acetaldehyde. We therefore concluded that acetaldehyde is indeed an oviposition attractant, not merely a stimulant.

Conclusion. The conventional chemical ecology approach for the identification of semiochemicals from natural sources is based on isolation of active ingredients after fractionation and bioassays. Here, we identified a potent oviposition attractant, acetaldehyde, using a reverse chemical ecology approach. We attempted to deorphanize OR highly expressed in the antennae of the southern house mosquito. Although all tested receptors failed to respond to our panel of 230 odorants, including physiologically and behaviorally significant odorants, we serendipitously found that a receptor, CquiOR36, responded to an old sample of nonanal. Careful investigation of the "contaminants" in nonanal samples led us to acetaldehyde, which is derived from ethanol and generated the strongest responses that we have ever recorded from mosquito ORs. Cage oviposition and dual-choice assays demonstrated that acetaldehyde is an oviposition attractant in a wide range of concentrations. A major problem with oviposition attractants is that normally they are active only in a narrow range of concentrations and thus their formulations have limited applications in the field. Under field conditions, concentrations of oviposition attractants tend to fluctuate because of evaporation and rain leading to concentration and dilution of attractants, respectively. Because it is effective over three orders of magnitude, acetaldehyde has enormous potential for practical applications in surveillance traps aimed at monitoring *Cx. quinquefasciatus* populations and circulation of West Nile virus and other pathogens.

Materials and Methods

For additional information, see *SI Appendix, SI Materials and Methods*.

Insect Preparations and Behavioral Studies. Mosquitoes used in this study were from a laboratory colony of *Cx. quinquefasciatus* originating from adult mosquitoes collected in Merced, CA, in the 1950s (26) and kept at the University of California Kearney Agricultural Research Center, Parlier, CA. Specifically, we used mosquitoes from the University of California, Davis, colony, which was initiated about 6 y ago with mosquitoes from the Kearney colony. In Davis, mosquitoes were maintained at 27 ± 1 °C, 75 ± 5% relative humidity, and under a photoperiod of 12:12 h. For cage oviposition assays (9), 40–60 mosquitoes were used 4–5 d after they received one blood meal. Each cage received 3 mL of defibrinated sheep blood that was provided with an artificial feeding apparatus. Blood was provided by the University of California, Davis, Biological Media Services, catalog #4024. For dual-choice assays (25), both gravid and nongravid females were used. Nonblood-fed female mosquitoes were 5–7 d old, and blood-fed mosquitoes were in the same conditions as those used for oviposition assays. For dual-choice assay, mosquitoes were separated into aluminum collapsible field cages (30.5 × 30.5 × 30.5 cm) with green polyester covers (BioQuip) the day before the experiments and provided with sugar and water. For each test, 5–10 females were transferred to the release cage of the dual-choice olfactometer.

Expression Analyses. Tissues (antennae, maxillary palps, proboscises, and legs) from 300 blood-fed female mosquitoes (4–7 d old) and antennae from 300 sugar-fed males were dissected using a stereomicroscope (Stemi DR 1663; Zeiss) and collected in ice-cold TRIzol reagent (Invitrogen). To compare expression of CquiOR36 by blood-fed and nonblood-fed mosquitoes, groups of 5-d-old female mosquitoes were separated into two cages, with one group provided with two blood meals and the other fed only sugar. The same number of mosquitoes was collected from each of the two cages, 1, 4, and 6 d after the time of the blood meals. All extractions were made three times, that is, with three biological replicates. Soon after tissue collection, total RNAs from these samples were extracted using TRIzol reagent. After RNA was quantified on a NanoDrop Lite spectrometer (Thermo Fisher Scientific), cDNA was synthesized from 200 ng of equal amounts of RNA using iScript Reverse Transcription Supermix for RT-qPCR according to the manufacturer's instructions (Bio-Rad). Real-time quantitative PCR was carried out by using a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad) and SsoAdvanced SYBR Green Supermix (Bio-Rad). The *CquiRPS7* gene was used as reference. For primers, see *SI Appendix, SI Materials and Methods*.

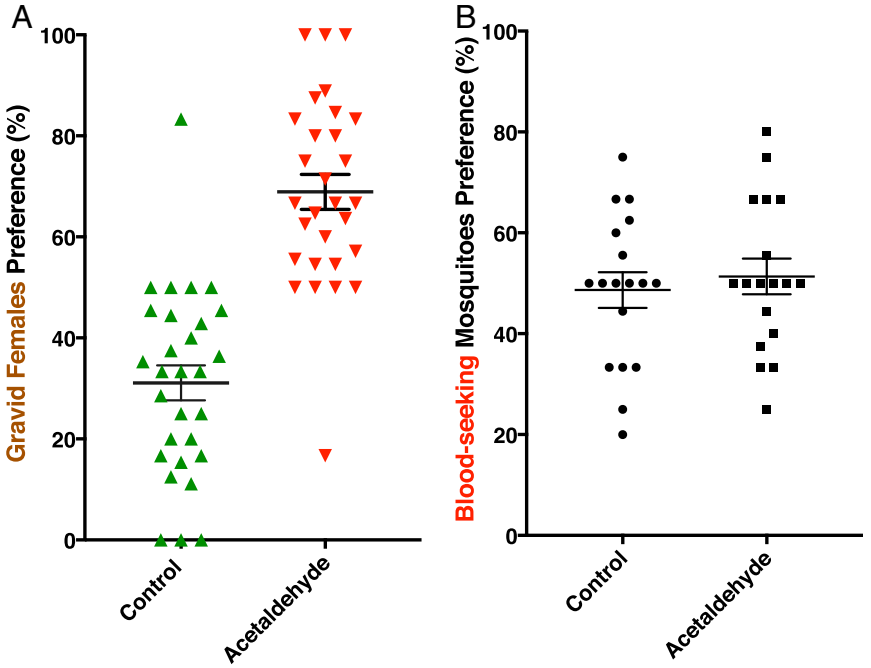

Fig. 5. Behavioral responses from southern house mosquito females in a dual-choice olfactometer in response to acetaldehyde (0.002%). (*A*) Response of gravid female mosquitoes, $n = 29$ releases, $P < 0.0001$. (*B*) Responses of blood-seeking (nonblood-fed) mosquitoes, $n = 18$ releases, $P = 0.7207$. Data were analyzed using the Wilcoxon matched-pair signed-rank test.

qPCR was performed with three biological replicates with each biological replicate replicated three times (i.e., three technical replicates per biological replicate). For comparative expression of the top 10 receptors, RNAs from female antennae and legs were used. For CquiOR36, qPCR was performed using antennae, maxillary palps, proboscis, and legs. Also, a comparison was made with RNAs from male and female antennae. The conditions for these reactions were denaturation for 3 min at 94 °C and then amplification for 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C for 25 cycles, followed by a final 5 min of 72 °C incubation time. PCR products were loaded onto 1% agarose gels and visualized on a UV light and photographed. Data were analyzed using the $2-\Delta\Delta CT$ method and normalized to *CquiRPS7* expression levels. The mean cycle threshold (CT) of the CquiOR36 gene in female and male antennae was normalized to the mean CT of the CquiOrco gene in the respective tissues. Data were calculated using 2-DDDCt. For comparison of the *CquiOR36* transcript in blood-fed and nonblood-fed mosquitoes, after normalizing with CquiRPS7, we normalized again all expression levels of each biological replicate to nonblood-fed mosquitoes 1 d after the time of the blood meal.

Cloning of CquiOR36, -55, -64, -93, -125, -132, and -151. Total RNA was extracted from 1,000 4- to 7-d-old female antennae with TRIzol reagent (Invitrogen). Antennal cDNA was synthesized from 1 μg of antennal total RNA using the SMARTer RACE cDNA amplification kit according to the manufacturer's instructions (Clontech). For primers, see *SI Appendix, SI Materials and Methods*. Of note, to clone CquiOR36, short-length primers (low annealing temperature, 43 °C) were used to prevent formation of hairpin structures. PCR products were purified by a QIAquick gel extraction kit (Qiagen) and then cloned into pGEM-T vector (Promega). Of 10 colonies selected, the ones containing the target plasmids (as indicated by PCR using gene-specific primers) were extracted by a QIAprep spin mini prep kit (Qiagen) and sequenced (Davis Sequencing). For subcloning into pGEMHE, see *SI Appendix, SI Materials and Methods*.

Electrophysiology. The tested animals were selected from F1 progeny for *Drosophila melanogaster*, which carried both UAS-CquiOR36 and DmelOrco-Gal4 promoter (24) and nonblood-fed, 2- to 3-d-old female mosquitoes. The EAG apparatus (Syntech) was linked to a computer with an EAG2000 data acquisition interface. Recording and indifferent electrodes were made of silver wires enclosed in drawn glass capillary needles, which were filled with 1 M potassium chloride in 1% polyvinylpyrrolidone. The reference electrode was inserted in the eye of an immobilized insect, and by using a micromanipulator MP-12 (Syntech), we placed the recording electrode on the third segment of a fruit fly antenna or at the tip of the last segment of a mosquito antenna. Compounds used as stimuli were freshly dissolved in paraffin oil and loaded onto filter paper strips (1 cm^2), which were placed into Pasteur pipettes. The preparation was bathed in a high-humidity air stream flowing from a Stimulus Controller CS-55 (Syntech) at 160 mL/min to which compensatory flow or stimulus pulse (125 mL/s, 300 ms) was added. Signal from the antenna induced by stimulus or control puff was recorded for 10 s.

Two-electrode voltage-clamp technique was performed as previously described (9, 15, 16, 22, 23). Briefly, the capped cRNAs were synthesized using pGEMHE vectors and an mMESSAGE mMACHINE T7 Kit (Ambion). Purified OR cRNAs were resuspended in nuclease-free water at 200 ng/mL and microinjected with the same amount of CquiOrco cRNA into *Xenopus laevis* oocytes in stage V or VI (purchased from EcoCyte Bioscience). Then the oocytes were kept at 18 °C for 3–7 d in modified Barth's solution [NaCl 88 mM, KCl 1 mM, NaHCO3 2.4 mM, $MgSO_4$ 0.82 mM, Ca(NO3)2 0.33 mM, CaCl2 0.41 mM, Hepes 10 mM, pH 7.4] supplemented with 10 mg/mL of gentamycin and 10 mg/mL of streptomycin. Odorant-induced currents at a holding potential of −80 mV were collected and amplified with an OC-725C amplifier (Warner Instruments), low-pass-filtered at 50 Hz, and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1440A and pCLAMP 10 software (Molecular Devices). For the panel of odorants, see *SI Appendix, SI Materials and Methods*.

Chemical Analyses. For chemical synthesis, see *SI Appendix, SI Materials and Methods*. Gas chromatography-mass spectrometry was performed on a 5973 Network Mass Selective Detector linked to a 6890 GC Series Plus+ (Agilent Technologies). The GC was equipped with an HP-5MS capillary column (30 m × 0.25 mm; 0.25-μm film; Agilent Technologies), which was operated with the following programs: 70 °C for 1 min, subsequently increased at a rate of 10 °C/min to 270 °C, and held at this final temperature for 10 min.

Injector was operated at 250 °C in pulsed splitless mode. MS transfer line was set at 280 °C, and the MS quad and MS sources were set at 150 °C and 230 °C, respectively. SPME analysis was done with 50/30 divinylbenzene/carboxen/polydimethylsiloxane (catalog # 57328-U; Supelco). Volatile compounds were collected from the headspace of the samples placed in a closed glass container with a rubber septum on the top. SPME analyses were performed with the oven starting at 35 °C for 5 min, raised to 70 °C at a rate of 2.5 °C/min, then raised at 5 °C/min to 150 °C, and subsequently raised to the final temperature of 250 °C at 10 °C/min. Under these conditions, acetaldehyde peak appeared at 1.48 min followed by ethanol peak at 1.59 min.

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